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Preparing and Mounting Bacteria.

By Prof. T. J. Burrill, Ph. D., F. R. M. S.

In my work I have not attempted to compare the results of the various new staining materials, many of which I have not seen, but what follows is some account of ordinary laboratory manipulation with common analine colors, such as can be obtained at any drug store for the coloring agents. Want of sufficient chemical knowledge has made it impossible for me to prosecute any systematic experimentation for the accomplishment of new results, and the pressure of other duties has been too great for the record of such achievements as should be presented in a paper of this kind. I can, therefore, only beg the indulgence of my fellow-workers whose practice has been more considerable and whose attainments are much more commanding than my own. A full account of my attempts to properly stain bacteria would be the record of more failures than successes, judged from my own estimate of what the latter should be, and this, not only at the outset of experimentation on several species, but more or less throughout the work. It is true there are species that may be very readily stained with scarcely a failure, by the use of common aniline, violet ink and other dyes, and this, too, without after-washing, leaving the material in which the organisms are imbedded too deeply colored; but what seems altogether satisfactory for temporary examination in these cases does by no means answer for all purposes nor with all organisms. The stain is not effective, or not suitable, or not permanent, or defective in one or more of several other particulars.

I enumerate some elements of successful staining, as follows:

1. The organisms should be decidedly and conspicuously colored. 2. The general mass of imbedding material should be

left unstained, or so different in color that the organisms can be distinctly seen. 3. There should be no granular or other precipitations from the staining material, nor should any portion of the latter remain as a coating on the glass. 4. The color should be suitable for the purposes required, and permanent, if the object is to be mounted for the cabinet. 5. The process should be as simple as possible and free from manipulative difficulties.

No one staining material, nor any single method of procedure can be made to answer well these requirements for all kind of bacteria, and in spite of successful practice upon many kinds the operator must ever be prepared for failure in some one or more of the specified particulars. Still, the failures after a little experience may be accounted the exceptions and success the rule.

Except for a few special results, aniline dyes are by far the most serviceable in staining bacteria; but these dyes are very different in their effects, and require different treatments. Probably an aqueous solution of methyl violet, so commonly used as ink, has been more generally used for the detection and examination of bacteria than any other agent, though in the hands of some of the most skillful workers gentian violet and methylene blue have each been pronounced most highly serviceable for general purposes. Ordinary violet is, when effective, usually quick and intense, and there is from the aqueous solution no sedimentary deposits consequent upon evaporation or subsequent washing with water, and most often the color of the mass can be suitably reduced by causing a stream of water to play from a wash bottle upon the material. There are, however, two objections to this stain, aside from the instances in which it entirely fails to color the objects. It is not suited for photography, and it is apt to be very fleeting, especially if exposed to the light. For the first there is no remedy, but for the second something can be done. The stronger the dye or the longer its application, especially the former, the more permanent the color, and sufficient washing may be practiced to secure the required tint of the field. After, however, the use of the dye of any strength, the application of alcohol is sure to discharge the color from everything; hence mounting in balsam is considered impossible. Treated with acetate of potash, the color is more stable and holds good for some months, but gradually fades in most cases. I have recently used instead of the aqueous solution of this violet aniline a solution in glycerine, with, so far, very satisfactory results. This may not be new to others—probably is not—but was to myself when first tried, about two months ago. After my experience with the aqueous solution, I was surprised to witness the permanence of the stain under treatment with alcohol and of exposure to full sunshine for several days. The preparations are still in good condition, though nothing further can now be stated as to the length of time they will so remain. The aniline is soluble in glycerine to any extent, as is the case with most of the other aniline colors tried, and was used of a strength of about one to five in bulk. To this may be added a small quantity of pure carbolic acid without detriment, so far as observed, and with advantage in the way of effectiveness in some cases. There is no difficulty whatever in passing the stained material through alcohol and benzole, or oil of cloves, into balsam; but in the case of bacteria dried on the cover-glass these preliminary immersions are unnecessary. The mounting is direct in pure balsam, care being taken that the prepared material is thoroughly dry. The color stands under subjection to the heat necessary for hardening the balsam. In the case of sections containing organisms, clearing agents are required, and may be freely used with at least certain species of bacteria—e. g., those commonly found on the mucous surfaces of the human body. I have not noticed this difference in permanence of color in the case of other anilines in glycerine; neither do I esteem the solutions in the latter always better than those in water or in alcohol, but in all cases in which the glycerine solutions are as effective they are to be preferred to others on account of the ease of handling. The alcoholic dyes are much inclined to creep or run over or up the surfaces of the containing dishes, and he is an expert workman who can leave his practice with clean fingers, to say nothing of forceps, needles, and perhaps of the work-table. Besides this, the alcohol on exposure rapidly evaporates, and often precipitations and sedimentary deposits mark the results. I do not know how others avoid this escape of alcohol when either on account of the necessities of the stain or of other business it must be left many hours. I have nothing better than a nest of ordinary porcelain dishes, the edges of which when necessary are smeared with glycerine. Stoppered bottles are not convenient. For the aqueous solutions these dishes are all that can be desired, unless intense heat is to be applied, when watch glasses take their place; but with the watery colors and much more so with the glycerine solutions, nothing is needed for a staining vessel. The dye is simply placed in a little pool on the prepared cover-glass, and the latter is held in pliers, or placed on a level surface, where, in the case of the glycerine, it may be left any length of time if protected from the dust. When ready it may be taken again in the pliers, washed by a stream from a wash-bottle with either water or alcohol, or both, without during the process losing account of the surface of the glass on which the material exists. If a second glycerine color is to be applied, this can be done at once in the same way without stopping for drying or for other preliminaries.

Suppose one wants to examine the organisms in his own or any other person's mouth. He may proceed as follows: Secure a little mucus from the tongue or teeth, taking care to avoid the remnants of the last bread and butter enjoyed. Place the substance on a clean glass slip, and if necessary mix by stirring. Make a little spatula of wood a quarter of an inch wide and cut square at the end, with which smear a well-cleaned cover glass, after the manner of spreading blood by drawing the spatula with the material once or twice, side by side, over the desired surface, holding the instrument at 30 or 40 degrees from the horizontal. Dry in the air or with moderate heat; then pass the glass through a flame somewhat quickly three or four times, holding the smeared surface up, and from a bottle, through the cork of which passes a glass rod, put on a drop of the glycerine violet; after one minute or less wash with water by means of a stream from a wash-bottle, and mount while wet for examination, the upper surface being wiped dry. If for permanent preservation, see that the cover glass and stained material is thoroughly dry; then mount directly in pure balsam, or, better in some respect's, but not so good in others, in Farrant's solution of gum and glycerine. All this can be done without putting the object out of hand, and in a very short time, and our specimen remains fit for the cabinet or for re-examinations months and probably years afterward. The use of an alcoholic solution of blue or red aniline would, with the same or similar treatment, be a complete failure, the whole surface, glass and all, remaining smeared with the dye in spite of water washing, and the whole color disappearing with the use of alcohol. With other organisms, as, for instance, the micrococcus of pear blight, the aqueous solutions of red or violet aniline are partially successful, while methylene blue entirely fails, but the strong glycerine solutions of the two former give far better results.

A method of "fixing" aniline colors, devised. I believe, by H. A. Reevis, of England, has been tried by myself with successful results, though sufficient time has not elapsed to prove the durability of the stain. Alcohol does not remove it. The stained material, whether section of tissue or dried film on the cover glass, is first treated with a saturated aqueous solution of tannin and distilled water in equal parts, to which is added a little carbolic acid, for three to five minutes; then, after washing in water, submit to a strong aqueous solution of potassio-tartrate of antimony (tartar emetic) for an equal time, and after thorough washing in water transfer to alcohol, a clearing oil, and balsam. I have tried a number of processes for staining *Bacillus tuberculosis*, the now well-known organism of pulmonary consumption; but have succeeded only with the use of aniline oil and fuchsin in alcohol and water, as recommended by Ehrlich and others. In one instance only a good stain, apparently, resulted from this red aniline with aniline oil in glycerine; but I could not repeat the accomplishment, possibly, however, from the want of fresh material though with that tried the Ehrlich process succeeded. Dr. Gradle, of Chicago, succeeds with a saturated alcoholic solution of fuchsin in five to eight times the quantity of a 5 per cent. aqueous solution of carbolic acid. I have tried the same mixture without success, but my proportion of carbolic acid was considerably greater. Gibbes, of England, makes a distinction between fuchsin and magenta, but no such difference is recognized by dealers in our country. A request for red aniline, or either of the above, is responded to by offering the same thing. Babes, of France, recommends a saturated aqueous solution, prepared with heat, of aniline violet 1 B. and an exposure of twenty-four hours, or the same with gentian violet. He further also modifies Ehrlich's method by making a saturated aqueous solution of aniline oil, using heat. To this is added 5 per cent. of absolute alcohol and 5 per cent. of a saturated alcoholic solution of fuchsin, or of a very concentrated solution of methyl violet 1 B. Twenty-four hours are again allowed for the stain, decolorizing with nitric acid and water one part to four. I have prepared the dye used by myself as follows: Into twenty grammes of 85 per cent. alcohol stir three grammes of aniline oil, and add this slowly while stirring to two grammes of pulverized crystals of fuchsin, the red aniline of the shops. To this solution add while constantly stirring twenty grammes of distilled water and bottle without filtering. This stain keeps well for some months, but gradually becomes unfit for use by the precipitation of granules of coloring matter. Doubtless this could be prevented by a little experimentation, thus making it possible to keep the dye in stock. In practice I have smeared a cover glass with a little sputum, dried and heated it as before described, and, having poured a little of the dye into one of a nest of porcelain dishes, have therein immersed the cover glass, perhaps several of them, put a little glycerine around the edge of the dish, and covered with another dish. This is left from two to twenty-four or even more hours when the cover glasses are taken out and freed from the adhering dye by washing in alcohol, usually by a stream from a wash-bottle, then immersed in nitric acid and water, one part to four, until all color perceptible to eye disappears, or usually about one minute. Wash thoroughly in water, and mount in water for examination. The special organisms should appear as minute ruby-colored rods, in a white field. If desired, the latter is stained blue or green by these anilines in glycerine by one minute's application. If for a permanent preservation, after drying mount by placing a minute drop of pure balsam on the center of a slide, and invert the cover glass upon it. Harden with moderate heat. These organisms can be seen by sharp eyes without staining, and by becoming familiar with their appearances, one can pretty certainly pronounce upon their presence without any preparation, provided they are numerous in the specimen examined, but as they alone hold the stain by the above process, it is not only easier to detect them when colored, but to more certainly know them from other kinds of bacteria very commonly found in sputa of the healthy, as well as the diseased.